CYTOPLASMIC DNA IN SEA URCHIN OOGENESIS STUDIED BY ³H-ACTINOMYCIN D BINDING AND RADIOAUTOGRAPHY

H. ESPER ENESCO AND K. H. MAN

Department of Biological Sciences, Sir George Williams University, Montreal, Quebec 43G 1M8 Canada

This study is concerned with the time at which cytoplasmic DNA of the sea urchin oocyte replicates in relation to the stage of oocyte development. Oocytes at many different states of development, as well as mature eggs, are found in mature sea urchin ovaries. They range in size from the smallest primary oocyte to the largest terminal oocytes. Since all different stages of oocyte development are present in the ovary, the events of oogenesis may be studied in a sequential manner.

The method chosen to detect the presence of cytoplasmic DNA in relation to the stage of oocyte development was ³H-actinomycin D incubation of sectioned material, followed by radioautography. It is well established that actinomycin D binds specifically to DNA (Muller and Crothers, 1968) and that this binding occurs on tissue sections (Ebstein, 1967, 1969). Once the ³H-actinomycin D binds specifically to DNA on the section, the location of the ³H-actinomycin D–DNA complex can be detected by radioautography. We thus have a method for localizing all nuclear and cytoplasmic DNA in tissue sections in relation to cell structures. This method allows us to compare the amount of cytoplasmic DNA detected by ³H-actinomycin D binding at progressive stages of oocyte development.

MATERIALS AND METHODS

Living supplies of the purple sea urchin, *Strongylocentrotus purpuratus*, were obtained from Pacific Biomarine Company, Venice, California. Ovaries of mature females were fixed in Carnoy's solution (ethanol : acetic acid, 3 : 1) for two hours. They were passed through graded alcohols, cleared in toluene and embedded in paraffin. Sections were cut at 5 microns, deparaffinized in xylol and hydrated through a graded alcohol series.

Control enzyme and TCA extraction of DNA and RNA

Controls were necessary to check the specificity of the ³H-actinomycin D binding under the experimental conditions used. Controls consisted of slides from which all DNA had been extracted prior to incubation with ³H-actinomycin D, either by treatment of the slides with DNase, using the method of Deitch (1966) or by extraction with hot trichloroacetic acid (TCA), using the method of Pearse (1961). As an additional control, RNase extractions, using the method of Amano (1962) were performed on another series of slides. The specificity of the above

extraction procedures was confirmed by Azure B staining for DNA and RNA using the cytochemical staining method of Flax and Himes (1952).

Incubation with ³H-actinomycin D

To determine the location of DNA in the oocytes, slides were incubated in ³H-actinomycin D. The method of Ebstein (1967, 1969) as modified by Aczel and Enesco (1973) was used. This method consists of incubating the slides with ³H-actinomycin D (5 μCi/ml) for two hours in a moist chamber in the dark, then rinsing the slides in distilled water and in a graded alcohol series to 70% ethanol to remove any ³H-actinomycin D which is not specifically bound to DNA. The ³H-actinomycin D (specific activity 3.7 Ci/mM) was obtained from Schwartz-Mann Company, Orangeburg, New York.

Radioautography and staining

Slides were air dried following ³H-actinomycin D incubation to prepare them for radioautography. The slides were then dipped in Kodak NTB2 liquid nuclear track emulsion, using the method of Kopriwa and Leblond (1962). After sufficient exposure (10 days) they were developed in D170 developer (Young and Kopriwa, 1964). The slides were then stained following radioautographic development with either cresyl violet or toluidine blue. The staining procedures followed were those selected by Thurston and Joftes (1963) for their compatibility with the radioautographic emulsion. After staining, the slides were dehydrated and mounted in Permount.

Staging of oocytes

The dimensions of the oocyte nucleus and cytoplasm as sectioned through their largest diameter were measured with the aid of a calibrated ocular micrometer. On the basis of these measurements, oocytes were classified into ten numerical stages, as described in the results section. Numerical stages 1 and 2 correspond to primary oocytes, stages 3 to 6 are growing oocytes and stages 7 to 10 are terminal oocytes using the classification defined by Cowden (1962) and by Esper (1965). At least 10 measurements were made to define each stage.

Histometrics and grain counts

Oocytes sectioned through their largest diameter were selected for this study. The nuclear and cytoplasmic diameter of these oocytes was measured, and the nuclear and cytoplasmic area was calculated. Using the measured values of nuclear and cytoplasmic diameter, nuclear volume and total cell volume were calculated. Cytoplasmic volume was then obtained by subtracting nuclear volume from total cell volume. The number of grains per $1000\mu^2$ cytoplasmic area was determined on the same sections used for histometrics. The grain counts, originally expressed in relation to area, were then transformed to express the total number of grains expected in relation to total cytoplasmic volume.

	Stage	No extraction	RNase extraction	DNase extraction	TCA extraction
Nucleus	1	266	282	8	2.9
	2	200	203	5.9	5.4
	3	130	153	0	0.5
Cytoplasm	1	66	77	4.5	1.7
	2	60	78	3.2	5.7
	3	43	48	2.8	1.4

RESULTS

It was first necessary to establish that ³H-actinomycin D was binding specifically to DNA under our experimental conditions. As would be expected, radioautographic examination showed that ³H-actinomycin D labelling was heavy over nuclei at all stages of oocyte development. Label appeared over the cytoplasm as well. Evidence of specific ³H-actinomycin D binding to DNA in both nucleus and cytoplasm of the sea urchin oocytes is presented in Table I. This table presents the number of silver grains per 1000 μ² over nuclei and over cytoplasm in the radioautographs, which in turn represents the amount of ³H-actinomycin D binding to DNA. As control data, grain counts from slides extracted with RNase, DNase, and hot TCA are presented. The grain count data for the RNase extracted slides is not significantly different from the non-extracted slides. Thus, RNase extraction does not influence ³H-actinomycin D binding. In contrast, on slides where all DNA has been removed by extraction with DNase or with hot TCA, the grain counts were reduced to very low levels, not significantly above background. This data clearly indicates that there is no ³H-actinomycin D binding in the absence of DNA. The effectiveness of the RNase, DNase and TCA extractions was further confirmed by the absence of selective staining when slides treated by these extraction methods were stained with Azure B bromide, which stains DNA and RNA selectively. The pattern described above shows that no ³H-actinomycin D binding takes place when all DNA has been removed from the sections. Table I presents representative grain counts from stages 1 to 3. The data for stages 4 to 9 follow exactly the same pattern.

Table II presents the direct measurements of nuclear and cytoplasmic area used to define the 10 stages of oocyte growth. From this data, the total cytoplasmic volume at each stage of oocyte growth was calculated. The data on Table II shows that there is a 26 fold increase in cytoplasmic volume between stages 1 and 10.

We now turn to the ³H-actinomycin D binding data for the cytoplasm, the primary concern of this study. Table II presents the grain counts, representing ³H-actinomycin D binding to cytoplasmic DNA, in a standard unit measure of

grains per 1000 μ^2 cytoplasmic area. This value decreases markedly from stage 1 to 5, suggesting a dispersal of cytoplasmic DNA correlated with the marked increase in size of the oocyte between these two stages. In contrast, from stage 6 to stage 10 the grain counts per 1000 μ^2 remains almost constant, despite the continued growth of the oocyte.

Since the oocyte is a three dimensional sphere, we will now use the above data to obtain an estimate of the amount of ^{3}H-actinomycin D binding to cytoplasmic DNA in the total cytoplasmic volume. Table II shows the total cytoplasmic volume at each stage of oocyte growth. Table II also shows the amount of ^{3}H-actinomycin D binding calculated to occur in relation to total cytoplasmic volume. This latter figure provides a comparative estimate of the relative amounts of cytoplasmic DNA present at various stages. The data shows that there is a 4-fold increase in ^{3}H-actinomycin D binding to total cytoplasmic DNA during oocyte development.

At each stage of oocyte development we have now determined two variables: cytoplasmic volume, and total grain counts calculated in total cytoplasmic volume Figure 1 expresses each of these variables in relation to stage of oocyte development. The two ordinates of Figure 1 are drawn to separate and arbitrary scales, for the purpose of comparing the shape of the two curves. This manner of presentation emphasizes the points of similarity between the two curves. Figure 1 shows that the most rapid increase in the amount of cytoplasmic DNA (grain counts) occurs in the primary and young growing oocyte (stages 1 to 5). There is a stabilization in the amount of cytoplasmic DNA at stage 5, but no marked increase between stages 5 to 10. When the volume increase of the oocyte is plotted on the same graph for comparison, we see that the increase in cytoplasmic volume starts slowly from stages 1 to 3, then rises sharply from stages 3 to 5, plateaus briefly at the stages 6 and 7, and then increases steadily from stages 7 to 10.

During the first stages of oocyte growth, the plots for increase in cytoplasmic volume and for increase in cytoplasmic DNA appear quite similar. However, after

TABLE II

The numerical stages of oocyte growth are defined in relation to the size of the nucleus and cytoplasm. Grain count data is expressed in relation to unit cytoplasmic area, total cytoplasmic area and total cytoplasmic volume.

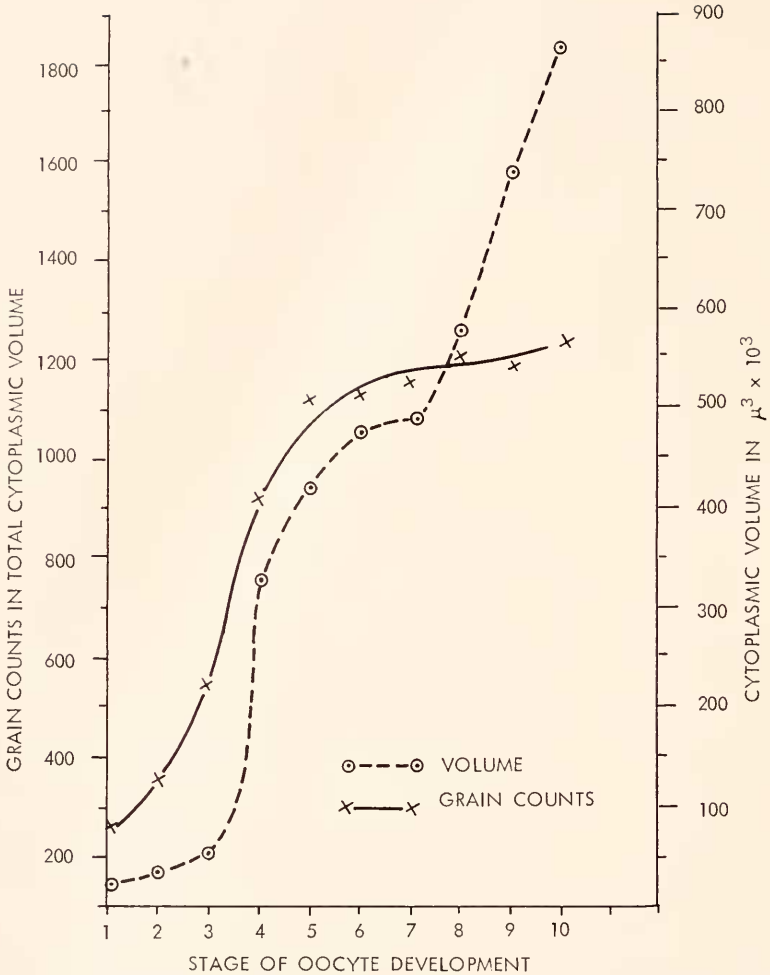| Stage	Nuclear area μ^2	Cytoplasmic area μ^2	Grain count per 1000 μ^2 cytoplasmic area	Cytoplasmic volume μ^3	Number of grains calculated per total cytoplasmic volume
1	110	900	266	24,300	362
2	135	1080	200	32,000	451
3	500	1250	130	49,100	652
4	850	5000	49	326,000	1096
5	900	6000	35	421,200	1208
6	1000	6500	29	483,800	1221
7	1100	6500	27	483,700	1253
8	1150	7200	25	573,200	1306
9	1200	9000	25	743,700	1289
10	1250	9500	25	867,900	1325

FIGURE 1. Grain counts on radioautographs, which represent the amount of ^{3}H-actinomycin D binding to cytoplasmic DNA at successive stages of sea urchin oocyte development are plotted on the left ordinate. Increase in cytoplasmic volume is plotted on the right ordinate. Arbitrary scales were chosen for the purpose of comparing the shape of the two curves. This comparison shows that increase in mitochondrial DNA parallels increase in cytoplasmic volume only in the early and growing oocyte stages.

stage 7 all similarity disappears. The oocyte continues to increase in volume, but there is no further increase in cytoplasmic DNA.

DISCUSSION

No controlled *in vitro* studies of ^{3}H-actinomycin D binding to sea urchin oocytes had been carried out prior to the present study. *In vivo* incubation studies

by Greenhouse, Hynes and Gross (1971) have shown that actinomycin D inhibits RNA synthesis in developing sea urchin embryos, and have established that ^{3}H-actinomycin D readily penetrates into living embryos. Radioautographic localization of the ^{3}H-actinomycin D binding sites showed that silver grains were distributed over the cytoplasm as well as over the nuclei of the early cleavage embryos. The authors interpret the cytoplasmic label as cytoplasmic DNA. Their experimental design did not permit confirmation with DNase extracted controls.

The amount of ^{3}H-actinomycin D binding which takes place in any experimental system is a function of both of the concentration of the ^{3}H-actinomycin D used and of the incubation time used (Simard, 1967). While ^{3}H-actinomycin D binding does not quantitatively reflect the absolute amount of cytoplasmic DNA present in the oocytes, we can use the actinomycin D binding data as a comparative estimate of the relative amount of cytoplasmic DNA available for the ^{3}H-actinomycin D binding at the various stages of oocyte development. The lack of ^{3}H-actinomycin D binding after DNase extraction in this study excludes any label being non-specifically bound to either nucleus or cytoplasm. Binding of ^{3}H-actinomycin D to cytoplasmic DNA has been reported in only one previous study (Aczel and Enesco, 1973), although several investigators have reported on ^{3}H-actinomycin D binding to nucleolar DNA (Simard, 1967, Camargo and Plaut, 1967).

It was first reported by Hoff-Jørgenson (1954) that sea urchin eggs contained large amounts of cytoplasmic DNA. This cytoplasmic DNA was first shown to be mitochondrial DNA by Piko, Tyler and Vinograd (1967), who also reported that one third of the cytoplasmic DNA in the sea urchin egg was associated with the yolk spherule fraction. These findings were subsequently revised by Berger (1968) who showed that DNA becomes associated with the yolk fraction in sea urchin eggs only as a result of contamination by the mitochondrial fraction. The results of Berger (1968), thus show that the "cytoplasmic" DNA of the sea urchin egg is entirely mitochondrial DNA. Dawid (1972) also cites evidence that the cytoplasmic DNA reported to occur in eggs of various species is entirely mitochondrial DNA. This is true not only for sea urchin eggs, but for frog eggs (Dawid, 1966) and for eggs of *Urechis caupo* as well (Dawid and Brown, 1970).

Mitochondrial DNA from mature sea urchin eggs has been isolated and characterized by Piko and associates (Piko *et al,* 1967, Pilo, Blain, Tyler and Vinograd, 1968). The replication of mitochondrial DNA during sea urchin oogenesis follows the same molecular replication pattern generally reported for mitochondrial DNA (Matsumoto, Kasamatsu and Piko, 1973).

In the present study, the fixation and staining methods necessary for radioautography are not suitable for morphological observation of mitochondria. Even though we cannot correlate silver grains directly with mitochondria, it is clear from the work of Berger (1968) that the mitochondria are the only DNA containing organelles in the cytoplasm.

The results presented here show that increases in the amount of cytoplasmic DNA, as detected by ^{3}H-actinomycin D binding, are confined to the preliminary and growing oocyte stages. No increase in cytoplasmic DNA takes place in the

terminal oocyte stages. If we now interpret cytoplasmic DNA as mitochondrial DNA, how do these results correlate with our knowledge of mitochondria in oocytes?

We do know that the number of mitochondria in the sea urchin oocyte inceases markedly during oogenesis. Verhey and Moyer, (1967) report that there is an average of 393 mitochondria in a small oogonium, as compared to an average of 19,827 mitochondria in a mature oocyte. These figures are based on calculations from electron microscope data, and represent a 50 fold increase in the number of mitochondria from the earliest to the latest stage of oocyte development. This data does not tell us the time at which mitochondrial replication takes place, and provides no information about mitochondrial DNA.

These combined results of this and the other studies cited show that the synthetic activities of the sea urchin oocyte are sequentially arranged into specific stages of oocyte differentiation.

The primary and growing oocytes are characterized by intensive RNA synthesis and production of ribosomes (Verhey and Moyer, 1967) as well as by the increase in cytoplasmic DNA reported in this study. Intensive yolk synthesis takes place at later stages (Esper, 1962, 1965).

Dawid (1972) suggests that large numbers of mitochondria may accumulate in the egg as a storage product to support embryonic development during the rapid cleavage stages. Gustafson (1965) reports that the number of mitochondria remain constant in the sea urchin from the mature oocyte stage through gastrulation. It is clearly the oocyte mitochondria which support the early development and differentiation of the sea urchin embryo. The oocyte mitochondria, containing mitochondrial DNA are thus stored in the oocyte for distribution among the cells of the growing embryo. These results show that mitochondrial replication takes place primarily in the early oocyte stages.

Summary

The relative amount of cytoplasmic DNA in sea urchin oocytes was studied at ten successive stages of oocyte differentiation in the sea urchin, *Strongylocentrotus purpuratus*. The cytoplasmic DNA was selectively localized by means of ^{3}H-actinomycin D binding on 5 μ sections of the ovaries. The specificity of the ^{3}H-actinomycin D binding to DNA was established using DNase extracted control slides. The results show that cytoplasmic DNA increases continuously during the primary and growing oocyte stages. Although oocytes at later stages of differentiation continue to increase in volume, there is no increase in cytoplasmic DNA during these later stages. The cytoplasmic DNA observed in this study is interpreted as mitochondrial DNA.

LITERATURE CITED

Aczel, J. and H. E. Enesco, 1973. DNA content of normal and tumor cells in mice. *J. Cell Biol.*, **59** : 2a.

Amano, M., 1962. Improved techniques for the enzymatic extraction of nucleic acids from tissue sections. *J. Histochem. Cytochem.*, **10** : 204–212.

BERGER, E. R., 1968. A quantitative study on sea urchin egg mitochondria in relation to their DNA content. *J. Cell Biol.*, **39** : 12a–13a.

CAMARGO, P. E. AND W. PLAUT, 1967. The radioautographic detection of DNA with tritiated actinomycin D. *J. Cell Biol.*, **35** : 713–716.

COWDEN, R. R., 1962. RNA and yolk synthesis in growing oocytes of the sea urchin *Lytechinus verigatus*. *Exp. Cell Res.*, **28** : 600–604.

DAWID, I. B., 1966. Evidence for the mitochondrial origin of frog egg cytoplasmic DNA. *Proc. Nat. Acad. Sci. U.S.A.*, **56** : 269–276.

DAWID, I. B., 1972. Cytoplasmic DNA. Pages 215–226 in J. D. Biggers and A. W. Schuetz, Eds., *Oogenesis*. University Park Press, Baltimore.

DAWID, I. B. AND D. D. BROWN, 1970. The mitochondrial and ribosomal DNA components of oocytes of *Urechis caupo*. *Develop. Biol.*, **22** : 1–14.

DEITCH, A. D., 1966. Cytophotometry of nucleic acids. Pages 327–354 in G. L. Wied, Ed., *Introduction to Quantitative Cytochemistry*. Academic Press, New York.

EBSTEIN, B. S., 1967. Tritiated actinomycin D as a cytochemical label for small amounts of DNA. *J. Cell Biol.*, **35** : 709–712.

EBSTEIN, B. S., 1969. The distribution of DNA within the nucleoli of the amphibian oocyte as demonstrated by tritiated actinomycin D radioautography. *J. Cell Sci.*, **5** : 27–44.

ESPER, H., 1962. Incorporation of ^{14}C-glucose into oocytes of *Arbacia punctulata*. *Biol. Bull.*, **123** : 246.

ESPER, H., 1965. Studies on the nucleolar vacuole in the oogenesis of *Arbacia punctulata*. *Exp. Cell Res.*, **28** : 85–96.

FLAX, M. H., AND M. H. HIMES, 1952. Microspectrophotometric analysis of metachromatic staining of nucleic acids. *Physiol. Zool.*, **25** : 297–311.

GREENHOUSE, G. A., R. O. HYNES, AND P. R. GROSS, 1971. Sea urchin embryos are permeable to actinomycin. *Science*, **171** : 686–689.

GUSTAFSON, T., 1965. Morphogenetic significance of biochemical patterns in sea urchin embryos. Pages 139–292 in R. Weber, Ed., *Biochemistry of Animal Development, Volume 1*. Academic Press, New York.

HOFF-JØRGENSEN, E., 1954. Deoxyribonucleic acid in some gametes and embryos. Pages 79–90 in J. A. Kitching, Ed., *Recent Developments in Cell Physiology*. Colston Papers, Volume 7.

KOPRIWA, B. AND C. P. LEBLOND, 1962. Improvements in the coating technique of radioautography. *J. Histochem. Cytochem.*, **10** : 269–284.

MATSUMOTO, L., H. KASAMATSU, AND L. PIKO, 1973. Mitochondrial DNA synthesis in sea urchin oocytes. *J. Cell Biol.*, **59** : 221.

MULLER, W. AND D. M. CROTHERS, 1968. Studies of the binding of actinomycin D and related compounds to DNA. *J. Mol. Biol.*, **35** : 251–290.

PEARSE, A. G. E., 1961. *Histochemistry: Theoretical and Applied.* [2nd. Ed.] London, J. & A. Churchill, 998 p.

PIKO, L., A. TYLER AND J. VINOGRAD, 1967. Amount, location, priming capacity, circularity and other properties of cytoplasmic DNA in sea urchin eggs. *Biol. Bull.*, **132** : 68–90.

PIKO, L., BLAIR, D. G., TYLER, A. AND J. VINOGRAD, 1968. Cytoplasmic DNA in the unfertilized sea urchin egg: physical properties of circular mitochondrial DNA and the occurrence of catenated forms. *Proc. Anat. Acad. Sci. U.S.A.*, **59** : 838–845.

SIMARD, R., 1967. The binding of ^{3}H-actinomycin D to heterochromatin as studied by quantitative high resolution radioautography. *J. Cell. Biol.*, **35** : 716–722.

THURSTON, J. M. AND D. L. JOFTES, 1963. Stains compatible with dipping radioautography. *Stain Technol.*, **28** : 231–235.

VERHEY, C. A. AND F. H. MOYER, 1967. Fine structural changes during sea urchin oogenesis. *J. Exp. Zool.*, **164** : 195–226.

YOUNG, B. A. AND B. M. KOPRIWA, 1964. The use of the Gevaert NUC-307 nuclear emulsion for radioautography at the electron microscope level. *J. Histochem. Cytochem.*, **12** : 438–441.